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Award Number: DAMD17-03-1-0531

TITLE: The Role of a Novel Topological Form of a Prion Protein in Prion Disease

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REPORT DATE: July 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual (1 Jul 2003 - 30 Jun 2004)

4. TITLE AND SUBTITLE
The Role of a Novel Topological Form of a Prion Protein in Prion Disease

5. FUNDING NUMBERS
DAMD17-03-1-0531

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Prion diseases are fatal neurological disorders of humans and other mammals. Prion diseases show an unusual etiology: they can arise from genetically, from infection through prion-contaminated food products, or sporadically. Most (but not all) cases of prion disease are associated with a conformationally altered form of the prion protein (PrP) known as PrPSc. Several lines of evidence indicate that while PrPSc is the infectious molecule, it may not be the proximate cause of toxicity in prion disease. Several other candidates for such a toxic species have been proposed, including an altered topological form of PrP known as CtmPrP. Lines of transgenic mice engineered to express CtmPrP develop a spontaneous prion-like disease (including lines described in this proposal). Thus, extending our knowledge of the biology of CtmPrP will likely lead to important clues about how all prion diseases induce neurotoxicity. We have characterized the cell biology of CtmPrP in detail in cultured neurons, and show that its cellular trafficking differs from normal PrP. We have also learned that CtmPrP is much less toxic when expressed on a PrP null genetic background; this result has important implications for the mechanism of toxicity in prion disease.

14. SUBJECT TERMS	15. NUMBER OF PAGES
prions, transmembrane PrP, transgenic mice, neurotoxicity	5
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Richard S. Stewart
Progress Report 2004
Award no. DAMD17-03-1-0531

The overall goal of the research award is to examine the role of a toxic prion protein (PrP) species known as CtmPrP (for C-terminal transmembrane PrP) in prion disease, and to develop diagnostic assays for CtmPrP. We and others have shown that transgenic mice expressing mutations in the PrP sequence which favor the formation of CtmPrP develop a spontaneous neurodegenerative illness with many of the same characteristics of infectious murine scrapie. However, these mice do not produce any detectable protease-resistant prion protein (PrPSc). Thus, there appear to be multiple signals which can induce a common neurotoxic pathway in prion disease. The elucidation of this common pathway (and any differences between the two inputs) will represent an important advance in our knowledge of prion disorders.

Our recent progress has relied heavily on the characterization of Tg(L9R-3AV) mouse lines established in our lab and preliminarily described in the award application. The creation of these mice was designed to make only the CtmPrP isoform in the brain, unlike previously described mouse lines. We have shown that this combination of two PrP mutants (L9R in the signal sequence, and 3AV in the transmembrane domain) produced only CtmPrP in vitro and in transfected cell lines (such as CHO and N2A; Stewart et al., 2001). However, both topological and cell biological analysis has shown that neurons from these mice produce a roughly equal proportion of CtmPrP and PrPC. This surprising result yields new information of the mechanism of topological decisions made by neurons, and suggests that the CtmPrP/PrPC decision may be regulated by cell type or developmental stage. This decision pathway may be a fruitful avenue of future study.

We have shown that neurons from Tg(L9R-3AV) mice make CtmPrP, and we have made extensive use of cultured neurons from the cerebellar granule layer to characterize the cell biology of CtmPrP. Previous work had shown that the same PrP mutant expressed in CHO cells never exited the endoplasmic reticulum, unlike PrPC which traffics rapidly to the cell surface (Stewart et al., 2001). In cultured neurons, however, CtmPrP traffics not the endoplasmic reticulum but to the Golgi apparatus, where it is prominent at steady-state. Our working model suggests that CtmPrP does not reach the cell surface, based on experiments which removed the PrPC on the cell surface by phospholipase treatment. No other PrP was detected on the surface, which by subtraction would have been CtmPrP. However, we cannot rule out that CtmPrP reaches the surface but quickly recycles to an internal compartment. This work is the first extensive characterization of the cell biology of this PrP isoform, and has yielded new information which immediately suggests new hypotheses for its mechanism of neurotoxicity, which will be the subject of future experiments. This work has recently been submitted for publication (Stewart and Harris 2004)

Two different lines of Tg(L9R-3AV) mice develop illness on the PrnP +/+ genetic background. To our surprise, it appears that breeding the transgenic mice onto a PrnP null background is sufficient to "rescue" the illness. Several animals from the Tg(L9R-3AV) B/PrnP o/o line have been examined and show no symptoms and no neuronal loss out to 600 days, while Tg(L9R-3AV) B/PrnP +/+ animals show illness at 180 days, and

extensive neuronal loss at death (400 days). Preliminary data suggests that animals from the second transgenic line [Tg(L9R-3AV) C/PrnP o/o] are delayed in showing symptoms compared to Tg(L9R-3AV) C/PrnP +/+ mice. We have ruled out trivial explanations for this phenotype such as lack of transgene expression, and we have shown that the localization of CtmPrP to the Golgi is identical in either genetic background. These results strongly imply that the mechanism of CtmPrP-induced toxicity requires the presence of endogenous PrP. These results also have significant implications for other observations which suggest that PrP plays a neuronal survival role, at least under certain conditions. We hypothesize that CtmPrP causes illness by somehow subverting the normal protective function of PrPC. It will be of great interest to determine if PrPSc also causes illness by a similar mechanism (it is well established that PrPSc is not infectious or toxic to PrnP o/o mice). Several independent lines of research appear to be converging toward this hypothesis, and thus future studies with these mice may have broad implications for all forms of prion disease. This work has also recently been submitted for publication (Stewart et al, 2004).

We have previously shown that CtmPrP retains the N-terminal signal peptide, and used this fact to develop specific and sensitive assays for CtmPrP in cells and tissues. The first assay relies on the small but reproducible mobility difference between CtmPrP and PrPC on SDS-PAGE after deglycosylation. We examined brain homogenates from several lines of scrapie-infected mice, and find no evidence for the presence of CtmPrP by this assay (Stewart and Harris, 2003). Other researchers have suggested that the presence of PrPSc can somehow influence CtmPrP production, but we find no evidence for such an event in our hands. The second assay relies on the generation of an antibody to the N-terminal signal peptide of PrP, which specifically recognizes uncleaved PrP molecules such as CtmPrP, while not recognizing processed PrPC. This assay can recognize CtmPrP in transfected cells at a level of less than 2% of total PrP (Stewart and Harris, 2003). We examined both uninfected and scrapie-infected N2A cells and find no detectable CtmPrP in either cell line, in concordance with the SDS-PAGE assay described above. This assay also recognizes unprocessed PrP produced by other means, such as PrP that is not translocated and accumulates in the cytoplasm. This cytosolic PrP has been shown to be a potent neurotoxin in transgenic mice (Ma et al, 2002). We can make two further conclusions from our assay of scrapie-infected N2a cells:1) Cytosolic PrP cannot be translocated and then retrotranslocated to the cytosol, as others have proposed, since we know the N-terminal signal has never been cleaved (Drisaldi et al., 2003), and 2) scrapie infection also does not induce synthesis of cytosolic PrP (Stewart and Harris, 2003).

We proposed to further develop the anti-signal peptide (SP) antiserum to be used as a screening device for animals. This work has not proceeded as of yet. A preliminary attempt to generate mouse hybridomas recognizing this antigen was not successful. We had also proposed to generate anti-SP antibodies to other PrP species. This may not be necessary, since it has been shown that our anti-mouse PrP SP serum also recognized human cytosolic PrP (Roucou et al., 2003). While the existing serum is plentiful, it is not an inexhaustible resource, and has less than ideal avidity. However, given the lack of evidence for CtmPrP in known scrapie samples, the use of this serum for high-throughput screening does not seem to be a priority at this time.

An ideal system for studying the toxicity associated with CtmPrP would be a cell culture model, as proposed. We have not noticed any loss of viability in standard cell lines when transiently transfected with L9R-3AV PrP. However, several attempts to create stable transfectants have not succeeded; similar reports have been published with cytosolic PrP mutants (Ma et al.,2002). An explanation for this toxicity remains unclear. We have made extensive use of primary neurons from our transgenic mice to examine their efficiency in primary culture. Neurons from these mice do not appear to show any loss in viability compared to controls under standard conditions. We are in the process of using various chemical stressors to determine if they are more susceptible to cell death than controls. We have also succeeded in using transient transfection of primary neurons to express wild type PrP at detectable levels. This is an inefficient process (1-10% efficiency), but reliable marker genes (such as EGFP) are being used to identify transfected cells. We will attempt to drive expression of mutant PrP to sufficient levels to specifically kill transfected neurons, and then determine which cell death pathways are activated during this process.

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